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during Mouse and Avian Endocardial Cushion Morphogenesis

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The formation of endocardial cushions in the atrioventricular (AV) canal of the rudimentary heart requires epithelial-to-mesenchymal cell transformation (EMT). This is a complex developmental process regulated by multiple extracellular signals and transduction pathways. A collagen gel assay, long used to examine endocardial cushion development in avian models, is now being employed to investigate genetically engineered mouse models with abnormal heart morphogenesis. In this study, we determine interspecies variations for avian and mouse cultured endocardial cushion explants. Considering these observed morphologic differences, we also define the temporal requirements for TGFβ2 and TGFβ3 during mouse endocardial cushion morphogenesis. TGFβ2 and TGFβ3 blocking antibodies inhibit endothelial cell activation and transformation, respectively, in avian explants. In contrast, neutralizing TGFβ2 inhibits cell transformation in the mouse, while TGFβ3 antibodies have no effect on activation or transformation events. This functional requirement for TGFβ2 is concomitant with expression of TGFβ2, but not TGFβ3, within mouse endocardial cushions at a time coincident with transformation. Thus, both TGFβ2 and TGFβ3 appear necessary for the full morphogenetic program of EMT in the chick, but only TGFβ2 is expressed and obligatory for mammalian endocardial cushion cell transformation.

Key Words: **endocardial cushions; TGFβ; heart development; atrioventricular canal; epithelial transformation.**

INTRODUCTION

The high incidence of structural heart defects highlights the complexity of cardiac development (Hoffman, 1995). The morphogenetic process of endocardial cushion transformation and invasion that occurs in the atrioventricular canal and outflow tract of the rudimentary heart is critical for normal valve development and cardiac septation leading to a mature, four-chambered heart.

The process of endocardial cushion transformation demonstrates temporal and spatial specificity within the embryonic heart tube. Initially, an acellular extracellular matrix (cardiac jelly) separates the myocardial and endothelial layers of the primitive heart tube. In the atrioventricular (AV) canal and outflow tract regions of the heart, endocardial cushions form as the result of an expansion of extracellular matrix rich in hyaluronan and chondroitin sulfate proteoglycans. At a specific time in development, inductive signals derived from the myocardium activate endothelial cells in these regions to undergo an epithelial-to-mesenchymal transformation (EMT). Cell transformation within the endocardial cushions occurs as a subset of endothelial cells hypertrophy, disengage cell–cell adhe-

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sions, extend filopodia, and invade the extracellular matrix (Markwald *et al.*, 1979). Invading mesenchymal cells within the endocardial cushions contribute to the formation of the mitral and tricuspid valve leaflets and complete atrial and ventricular septation (Eisenberg and Markwald, 1995). This cardiac morphogenetic process is unique within the endocardial cushions, as adjacent atrial or ventricular endothelia fail to undergo transformation *in vivo* and do not appear to possess the capacity to transform *in vitro* even when exposed to appropriate inductive stimuli (Runyan and Markwald, 1983).

Progress in understanding the molecular regulation of AV canal development has been facilitated by an *in vitro* three-dimensional collagen gel culture system. Bernanke and Markwald (1979) developed an *in vitro* model of cardiac mesenchyme formation utilizing a hydrated type I collagen gel matrix. Subsequently, Runyan and Markwald (1983) modified this assay to demonstrate temporal correlation to *in vivo* events, regional specification within endocardial cushion tissues, and a dependence of this process on inductive signals from the cushion myocardium.

TGFβ2 and TGFβ3 are expressed in the endocardial cushions of avian embryos prior to transformation events and following EMT, while TGFβ1 has not been detected. In addition, distinct functional roles for both TGFβ2 and TGFβ3 (Boyer *et al.*, 1999; Nakajima *et al.*, 1994; Potts *et al.*, 1991) and their cognate TGFβ receptors (Boyer and Runyan, 2001; Brown *et al.*, 1996, 1999) have been elucidated by using the *in vitro* assay with avian endocardial cushion tissue. TGFβ2 signaling appears to mediate AV canal endocardial cell activation and separation, while TGFβ3 is obligatory for mesenchymal cell formation and invasion into the underlying matrix. In the developing mouse, however, apparent conflicting data have been reported concerning the requirements of TGFβ molecules during endocardial morphogenesis. Mouse embryos deficient for TGFβ1 or TGFβ3 do not have cardiac phenotypes (Letterio and Roberts, 1996; Sanford *et al.*, 1997), and while AV endocardial cushion morphogenesis is abnormal in TGFβ2$^{-/-}$ embryos, the most significant cardiac phenotype is attributed to disrupted outflow tract development (Sanford *et al.*, 1997). These data suggest that there are different TGFβ requirements during avian and mouse endocardial cushion morphogenesis.

To further explore the roles of TGFβ2 and TGFβ3 in mammalian cardiac valve development and to determine interspecies variation in the *in vitro* collagen gel invasion assay, we executed parallel studies with mouse and chick endocardial cushion tissues. Substantial interspecies differences were identified between avian and mouse AV canal explants which may be attributed to distinct growth factor requirements. In this regard, we determined that TGFβ ligands do not have the same specificity on AV canal morphogenesis during mouse development as they do during avian development. Only TGFβ2 has a developmental role in mouse AV endocardial transformation into mesenchyme that is independent of TGFβ3.

TABLE 1
Interspecies Comparison of Developmental Stage and Somite Number

Chick		Mouse	
Stage (HH)	Somite number	Stage (dpc)	Somite number
12	16	8	4
13	19	8.5	10–13
14	22	9	17
15	24–27[a]	9.5[a]	21–26
16[a]	26–28	10[a]	26–29[b]
17	29–32[b]	10.5	30–34
18	32–36	11	>34

Note. Because embryos develop at different rates under the same incubation conditions or within the same litter, somite number was utilized as an additional method for comparing mouse and chick embryos.

[a] AV endocardial cushion transformation observed *in vivo* between 21–28 somites for mouse and 25–28 for chick.

[b] Formation of endothelial cell monolayer at higher somite number at this stage.

EXPERIMENTAL PROCEDURES

Embryo Isolation and Staging

Mouse and chick embryos were dissected free of decidua and embryonic membranes in 4°C sterile 1× Tyrode's salt buffer and staged in a standard manner for avian Hamburger and Hamilton stages (Hamburger, 1992) and mouse (Kaufman and Bard, 1999). Two independent observers using a dissecting stereo microscope manually counted somites. Somite count and developmental stage of embryos are shown comparatively in Table 1. Embryos were used for *in vitro* analysis as described below.

AV Canal Endocardial Cushion Morphogenesis Assay

Fertilized White Leghorn chicken eggs (Rosemary's Farm, Santa Clara, CA) were incubated at 37°C for 48–72 h to obtain embryo stages encompassing the initial formation of the acellular endocardial cushion (St. 12) to the appearance of a large population of mesenchymal cells within the cushions (St. 18). Embryonic hearts were removed and the AV endocardial cushions regions dissected apart from the adjacent atria and ventricular chambers. The AV canal was opened longitudinally to expose the inner endocardial surface. AV canals were placed endothelial surface down onto hydrated type I collagen gels and allowed to attach for 6 h according to published procedures (Potts *et al.*, 1991). M199 media (Gibco/BRL, Rockville, MD), supplemented with 0.01% insulin, transferrin, and selenium (ITS) (Gibco/BRL) and 1% chick serum (Gibco/BRL) served as culture medium. Explants were cultured for 48 h at

TABLE 2
Experimental Conditions for Chick and Mouse Endocardial Cushion Explant Cultures

	Chick	Mouse
Gel casting (R T for 30 min)	Type I collagen (1 mg/1 ml) 100 μl 10× M199 100 μl 2.2%(wt/vol) $NaHCO_3$ 300 μl per 12 mm well	No modifications
Gel hydration (37°C, 5% CO_2 for at least 30 min)	300 μl M199 containing 1% chick serum, ITS, PS	300 μl OPTI-MEM 1% fetal calf serum, ITS, PS
Explant attachment	Remove media prior to placement. Incubate 6 h at 37°C, 5% CO_2	Remove media prior to placement. Incubate 12 h at 37°C, 5% CO_2
Explant culture	Add 300 μl supplemented M199. Culture 48 h at 37°C, 5% CO_2	100 μl M199 supplemented with 1% fetal calf serum, ITS, PS. Culture 48 h at 37°C, 5% CO_2

Note. ITS, insulin, transferrin, and selenium; PS, penicillin streptomycin.

37°C, 5% CO_2 prior to determining the extent of endocardial migration or spread and mesenchymal cell invasion by using Hoffman phase microscopy as previously described (Runyan and Markwald, 1983). Briefly, the criteria used for endocardial cell migration was the appearance of rounded, polygonal cells on the collagen gel surface. The primary morphologic criteria for mesenchymal cells invasion was the appearance of cells with characteristic stellate appearance within the gel matrix (Runyan and Markwald, 1983).

Mouse AV canal "explants" from wild-type FVB mouse embryos were isolated as described from timed fertilized female mice and somite number verified embryonic age. Experimental conditions were optimized for mouse endocardial cushion cultures to obtain similar morphologic events compared with the established avian system. Briefly, M199 media was consistently used for gel casting in four-well microculture dishes (Nalge Nunc, Naperville, IL). However, substituting OPTI- MEM media (Gibco/BRL) plus 0.01% ITS (Gibco/BRL) to hydrate the polymerized type I collagen gels before mouse explant placement provided reproducible cultures with minimal supplements (Camenisch *et al.*, 2000). Mouse tissues required 12 h for attachment to the collagen gel surface at 37°C, 5% CO_2 prior to the application of 0.1 ml of M199 supplemented with 1% fetal calf serum (Hyclone, Logan, UT), 50 U/ml penicillin and 50 μg/ml streptomycin, and 0.01% ITS (Gibco/BRL). Explants were cultured at 37°C, 5% CO_2 for 48 h. Table 2 details the differences in culture conditions between the two species.

Antibody Treatments

Stage 15/16 chick and E9.5 mouse endocardial cushion explants (AV canal and outflow tract) were allowed to attach to the collagen gel matrix as described. M199 media was supplemented with either 10 μl antibody to TGFβ3 (D3) (Developmental Studies Hybridoma Bank), antibody to TGFβ2 (1 μg/ml) (R&D Systems, Minneapolis, MN), or isotype control IgG or IgM (0.25 mg/ml). The anti-TGFβ2 antibody has less than 2% cross-reactivity to other TGFβ molecules (R&D Systems). Epitope mapping of the anti-TGFβ3 antibody recognizes the identical seven-amino-acid peptide sequence (RALDTNY) common to human and mouse isoforms (Boyer *et al.*, 1999). Explants were cultured in the continued presence of indicated antibody for 48 h. Independent experiments were repeated a minimum of three times. Transformation of endothelium to mesenchyme and invasion into the collagen gel was imaged by using Hoffman optics as described above.

In Situ Hybridization

Mouse embryos were removed from decidua and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (0.1 M; pH 7.2). Fixed embryos were dehydrated in graded ethanol and xylene and embedded in paraffin. Serial 5-μm sections were mounted onto TESPA-coated (Sigma, St. Louis, MO) glass slides. A 442-bp *Eco*RI/*Xho*I *TGFβ2*-cDNA fragment cloned into Sp72 (kindly provided by T. Doetschman) was linearized with *Xho*I or *Eco*RI and transcribed with T7 or SP6 RNA-polymerase to obtain sense and anti-sense ^{35}S-labeled riboprobes, respectively. A 609-bp *Sma*I/*Eco*RI, *TGFβ3*-cDNA fragment cloned into pGEM7zf+ (kindly provided by Dr. H.L. Moses, Vanderbilt University) was linearized with *Xba*I or *Hin*dIII and transcribed with SP6 or T7 RNA polymerase to obtain sense and antisense probes, respectively.

In situ hybridization was performed on PFA-fixed tissue sections as described by Hierck *et al.* (1996). After hybridization, sections were dehydrated in graded ethanol, air-dried, coated with Ilford G5 emulsion (ILFORD Ltd, Mobberly, England), and exposed for 14 days at 4°C. Following exposure, slides were developed in Kodak D19 developing solution (Kodak, Chalon s. Saone, France) for 4 min at room temperature, rinsed in distilled water, and fixed in Ilford Hypam fixative (ILFORD Ltd). Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with Malinol (SCHMID&CO, Stuttgart-Untertürkheim, Germany) prior to light microscopic examination.

Immunohistochemistry

Deparaffinized sections were briefly rinsed in PBS, followed by 15 min of treatment with 0.3% H_2O_2 in PBS. Sections were incubated overnight with primary antibody to α-smooth muscle actin (HHF-35) (DAKO A/S, Glostrup, Denmark) diluted 1:500 in PBS with 0.05% Tween 20 and 1% BSA at room temperature. Sections were thoroughly rinsed followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:200, 1.5 h, DAKO A/S) followed by goat-anti-rabbit immuno-

globulin (1:50, 1.5 h, DAKO A/S), rabbit-peroxidase-anti-peroxidase (1:500, 1.5 h, DAKO A/S), and subsequent treatment with 0.04% diaminobenzidine tetrahydrochloride/0.06% H_2O_2 in 0.05 M Tris–Maleic acid (pH 7.6) for 10 min at room temperature. Sections were briefly counterstained with Mayer's hematoxylin, dehydrated, and mounted with Entellan (Merck, Darmstadt, Germany).

RESULTS

Morphologic Assessment and in Vitro Comparison

Variation between chronological age and gestational/developmental age during embryogenesis is well recognized for incubated chick embryos and within mouse litters (Kaufman and Bard, 1999). Somite number was therefore utilized as an additional method for comparing developmental stages between species. Detailed staging of embryos, focused upon the appearance of phenotypic mesenchymal cells within the AV endocardial cushions, demonstrated that cushion cell transformation occurs at approximately St. 16/17 in the chick and ~E9.5 in the mouse. These morphologic stages encompass a range of somites (Table 1). The most consistent external measure for AV endocardial cushion transformation was the attainment of 26 (±1) somites in either species. Cell transformation within the outflow tract endocardial cushions appeared delayed by approximately 1 day in the mouse and one to two stages in the chick. Somite staging was used to verify embryonic age for direct comparisons of TGFβ expression patterns as well as analysis of functional data from parallel endocardial cushion explant cultures.

Direct comparisons of endocardial cushion explants cultured *in vitro* revealed important morphologic differences between mouse and chick tissues (Fig. 1). In avian AV canal explants, endothelial cells initially leave the explant and spread over the collagen gel surface as a monolayer prior to transformation *in ovo* (Runyan and Markwald, 1983). This activation step results in loss of cell–cell junctions and separation between adjacent cells. A subset of these cells subsequently extends pseudopodia and transforms into a mesenchymal cell phenotype capable of migration on and into the gel matrix (Figs. 1E and 1F). In contrast, mouse AV canal explant cultures established prior to EMT fail to display a clearly defined endothelial monolayer (Figs. 1A and 1B). Endothelial cell–cell separation, "activation," is not readily apparent in mouse AV canal explants. Rather, the mouse cardiac endothelia appear to transform directly, exhibiting characteristic mesenchymal morphology and invasive capability in close proximity to the primary explant (Fig. 1B). Cultured AV canal endocardial cushions isolated from chick or mouse embryos that had already undergone cell transformation *in vivo* (somite number 28 or greater) appear more similar. For these later developmental stages, AV canal explants from both species display an expansion of endothelial cells over the collagen gel and decreased mesenchymal cell formation and invasion (Figs. 1C, 1D, and 1H). Collectively, these observations define the temporal specificity of EMT during both chick and mouse AV canal morphogenesis *in vitro* (Table 1).

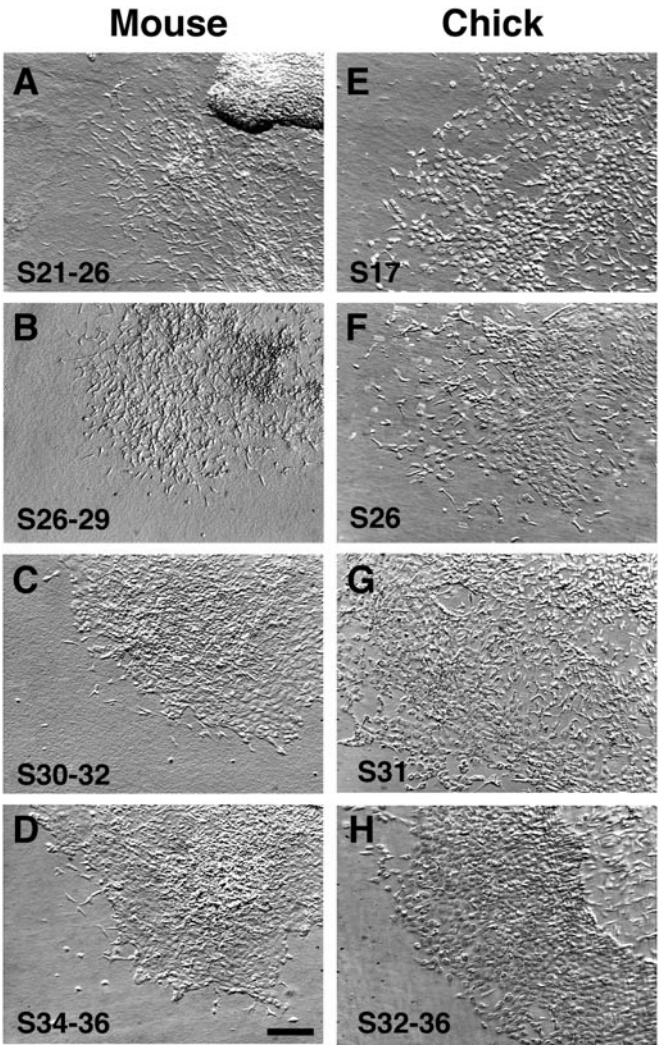

FIG. 1. *In vitro* analysis of endocardial cushion morphogenesis reveals important differences between mouse and chick AV canal explants. Mouse AV canal explants cultured through the period of *in vivo* transformation fail to display clearly defined endothelial cell spread over the collagen gel surface or cell–cell separation; invasive mesenchyme appear in close proximity to the explant site, suggesting an immediate transformation (A, B). Chick AV canal explant culture from similar stages demonstrates characteristic endothelial cell spread, cell–cell separation, and the appearance of transformed mesenchymal cells concentrated at the periphery of the endothelial sheet (E, F). "Post-transformation" AV canal explants exhibit a significant endothelial expansion over the gel surface for both the mouse (C, D) and chick (G, H). The mouse endothelial sheet in later staged explants does not display cell–cell separation evident in the chick explants. Scale bar, 45 μm.

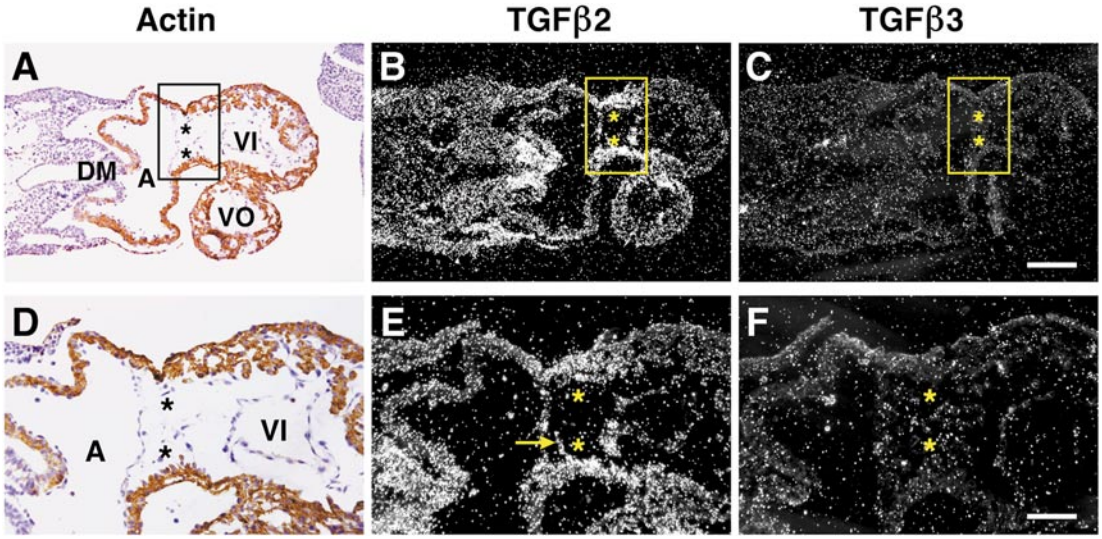

FIG. 2. Expression of TGFβ2 and TGFβ3 in ~E10.0 mouse embryos. Sections of the atrium (A) and ventricular inflow and outlet segment (VI, VO) of an E10.0 looped mouse embryo heart. The myocardium is positive for muscle actin (brown staining, A and D), while the endocardium and the AV canal cushions are negative (hematoxylin counterstained). Dark field images in the remaining panels show detection of TGFβ2 (B, E) and TGFβ3 mRNA (C, F). Note the high TGFβ2 expression in the AV canal myocardium (yellow asterisk) and the TGFβ2-positive cells invading the endocardial cushion (yellow arrow) and the dorsal mesocardium (DM). Scale bar in C, 200 μm and in F, 50 μm.

Expression of TGFβ2 and TGFβ3 during Mouse AV Canal Formation

The peptide growth factors TGFβ2 and TGFβ3 are both expressed in the avian endocardial cushions (Boyer *et al.*, 1999) and have distinct roles during EMT, but it is unclear whether they play similar requisite functions during mouse AV canal morphogenesis. The expression of both *TGFβ2* and *TGFβ3* was examined during the critical developmental period encompassing valvular formation, from E9.0–E13.0. Expression of *TGFβ3* could not be detected within the time span of endocardial cushion development and associated epithelial–mesenchyme transformation in the mouse embryo (E9.0–E10.0) (Figs. 2C and 2F). Expression of *TGFβ3* within the endocardial cushions was only detected after E11.0 (Fig. 3C), once mesenchymal cells have already invaded the matrix and the atrial septum has connected with the AV cushion tissue. In contrast, *TGFβ2* is highly expressed at E9.0–E10.0 in the myocardium lining the endocardial cushions (Figs. 2B and 2E) and persisted beyond E12.0 (Fig. 3B). The endothelium-derived mesenchymal cells, which begin to populate the AV endocardial cushions between E9.0 and E10.0, are *TGFβ2*-positive (yellow arrow, Fig. 2E). The dorsal mesocardium also highly expresses *TGFβ2* at E10.0. The endocardium of mouse AVC cushions appears to express *TGFβ2* but not *TGFβ3*. Expression of *TGFβ2* becomes enhanced over the next 3 days of cushion development concomitant with the increased number of mesenchymal cells that populate the cushions and subsequent remodeling into functional valves (Fig. 3). Thus, *TGFβ2* is expressed in the mouse cushion myocardium as well as in the invading mesenchymal cells during AV canal morphogenesis. These data show that TGFβ2, but not TGFβ3, is strongly expressed in the early cardiac endocardial cushions, and suggest that TGFβ2 is the key growth factor directing EMT within the mouse AV canal.

TGFβ2 and TGFβ3 Functions during in Vitro Endocardial Cushion Morphogenesis

Our *in situ* hybridization results concentrating on the E10–E12 mouse heart are consistent with previous TGFβ isoform expression studies (Dickson *et al.*, 1993). Combined with the primary phenotypes of the TGFβ-deficient animal models, these data suggest that TGFβ2, but not TGFβ3, has a role during EMT in the mouse. Specific neutralizing antibodies to TGFβ2 and TGFβ3 were therefore utilized in the collagen gel assay to compare the impact these growth factors have on the transformation of cardiac endothelium into invasive mesenchyme in mouse and avian endocardial cushions.

TGFβ2 blocking antibody inhibits endothelial cell–cell separation and decreased mesenchymal cell invasion as previously reported in avian explant cultures (Fig. 4B) (Boyer *et al.*, 1999). Similarly, neutralization of TGFβ2 abated phenotypic transformation in mouse AV canal explants, (Fig. 4A). In striking contrast to the immediate transformation observed in naïve E9.5 mouse endocardial

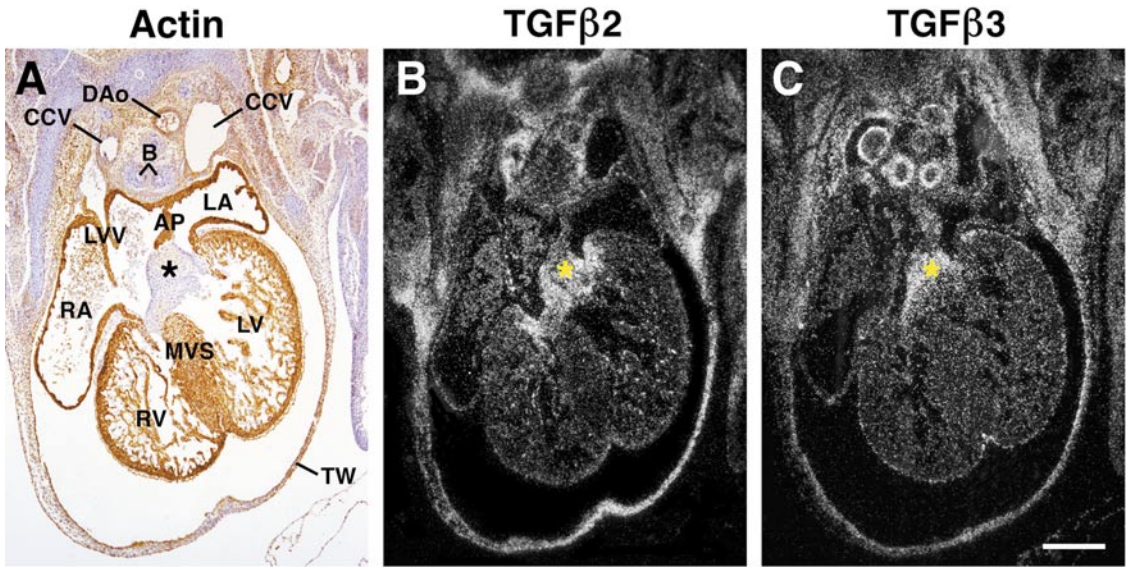

FIG. 3. Expression of TGFβ2 and TGFβ3 in E12.0 mouse embryos. Sections of an E12.0 mouse embryo heart at the level of the inflow of the left (LV) and right ventricle (RV) showing the four-chamber configuration. In this region of the heart, the actin-positive atrial septum (AP) and muscular ventricular septum (MVS) separate the atrium (LA, RA) and ventricle (LV, RV) and are both fused to the actin negative AV cushions (asterisk) (A). TGFβ2 expression is specific to the central AV endocardial cushion tissue, the myocardium of the AV canal, and to the forming leaflets of the AV valves (B). TGFβ3 is expressed in the AV endocardial cushion tissue to a lesser extent, but highly expressed in the bronchi (B) and common cardinal vein (C). DAo, descending aorta; TW, thoracic wall; CCV, common cardinal vein; LVV, leaflet of venous valve. Scale bar, 100 μm

cushion explant cultures (Figs. 1A and 1B; and Fig. 4E, control), neutralizing TGFβ2 caused a substantial outgrowth of endocardial cells on the gel surface and the appearance of some cell–cell separation, but no invasive mesenchyme within the gel compared to control mouse AVC explants (Fig. 4E).

Neutralizing antibody to TGFβ3 caused a profound inhibition of cell transformation in chick explants (Fig. 4D) as previously reported (Boyer *et al.*, 1999). In contrast, TGFβ3 antisera had little impact on EMT in mouse (Fig. 4C). Transformation into mesenchyme was indistinguishable compared with IgG-treated mouse AV canal explants (Fig. 4E).

In a similar series of experiments, the effects of TGFβ inhibition were examined in endocardial cushion explants derived from the outflow tract regions. Both mouse (Fig. 5E) and chick (Fig. 5F) outflow tract endocardial cushions exhibit EMT in the collagen gel assay. An inhibition of cell transformation was observed with TGFβ2 blocking antisera in mouse outflow tract explants (Fig. 5A), but little effect on cell–cell separation or transformation was observed in avian outflow tract explants (Fig. 5B). Similar to results observed in AV canal explants, TGFβ3 neutralization attenuated EMT in avian, but not to any substantial level in mouse outflow tract endocardial cushion explants (Figs. 5C and 5D). Collectively, these data define a functional role for TGFβ2 during mammalian endocardial cushion EMT and demonstrate that TGFβ3 is not required for transformation events during this developmental process in mouse development (summarized in Table 3).

DISCUSSION

The formation of the four-chambered heart requires precise interactions among multiple cell types. The high frequency of congenital heart defects involving abnormal cardiac valve formation and septation reflects the complexity of these developmental events. Among infants, congenital heart defects are the largest class of birth defects and account for one of the three most common causes of death in the first year of life (Rubin *et al.*, 1985). The relatively high occurrence and associated costs of congenital heart defects make a compelling case to better understand the molecular and cellular basis of heart development.

Distinct Temporal Events in Avian and Mouse Endocardial Cushion Morphogenesis

Initial progress in understanding development within the embryonic cardiac AV canal has been advanced with use of avian heart tissue in a three-dimensional collagen gel culture system. The ability to study *in vitro* phenotypic

changes analogous to *in vivo* morphogenic events in an experimental system has led to the current understanding of molecular events regulating endocardial cushion cell transformation, differentiation, and invasion. Further progress has been made by the creation of mouse models harboring genetic perturbations resulting in cardiac phenotypes; however, almost all experiments utilizing the three-dimensional collagen gel assay have been performed by using AV canal tissue derived from avian embryos. Although Sissman (1970) compiled observations describing developmental landmarks during cardiac morphogenesis among several species, no direct detailed comparison between mouse and chick endocardial cushion morphogenesis has been reported.

We and others investigating mouse AV canal formation have begun using the collagen gel assay to assess transformation and invasion (Camenisch *et al.*, 2000; Dor *et al.*, 2001; Lakkis and Epstein, 1998; Mjaatvedt *et al.*, 1998). Here, we report staging of avian and mouse embryos based on structure and somite number, which provides the most accurate comparative measure of endocardial cushion morphogenesis between species. We were able to discern normal interspecies variations by directly comparing parallel AV canal explants derived from equivalent somite staged mouse and chick embryos. Specifically, while cell transformation and invasion occurs in both mouse and chick AV canal explants at ~26 somites (±1), there is an absence of discernible endothelial cell migration in mouse-explanted tissue at this stage. In contrast, cardiac endothelium from comparable chick AV canal explants cultured for 48 h displays a characteristic spread onto the collagen gel surface and demonstrates cell–cell separation prior to mesenchymal cell transformation (Runyan and Markwald, 1983). Utilization of anatomic structure and somite number for assessing developmental progression will be important for future investigations of heart septal and valve formation, particularly for analysis of mutant embryos displaying complex cardiopathies and growth retardation.

Post-Transformation Events

There is a dramatic change in AV canal explants cultured from later staged embryos that have already executed epithelial-to-mesenchymal transformation within the endocardial cushions. In both mouse and avian AV explants at 30 somites and greater, an extensive endothelial monolayer spreads onto the collagen gel surface. Transformation still occurs, but at a diminished level compared with earlier stages. This observation suggests that, following *in vivo* cushion endothelial cell transformation, there is a shift toward endothelial expansion and cessation of transformation. This may reflect increased VEGF levels which appear to serve a multifactoral role during endocardial cushion morphogenesis (Dor *et al.*, 2001). VEGF acts as a negative regulator of transformation and a mitogen for the temporary expansion of the endothelium to fill gaps vacated by activated endocardial cells. This activity coincides with diminished *TGFβ2* expression at E11.5 in the myocardium of the AV canal (Dickson *et al.*, 1993) and, as we show here, the detectable expression of *TGFβ3*. In this regard, VEGF protein production is increased by TGFβ3 in a dose-dependent manner (Saadeh *et al.*, 2000), suggesting that TGFβ3 may be upstream of VEGF as required for terminating transformation activity. The observed phenotypic similarities in mouse and chick endocardial cushion tissues following endothelial cell transformation suggest that both induction of VEGF and a shift in TGFβ expression may be required for successful remodeling of the cushions into differentiated valvular tissue. Collectively, this direct interspecies comparison in the collagen gel invasion assay has defined the developmental window of endocardial cushion EMT and revealed distinct temporal variations between avian and mouse AV canal morphogenetic events.

Temporal Requirement for TGFβ2 in Mouse Endocardial Cushion Mesenchyme Formation

In avian models, the peptide growth factors TGFβ2, signaling through the Type III TGFβ receptor, and TGFβ3, through the Type II TGFβ receptor, direct nonredundant signaling cascades in the developing AV endocardial cushions (Boyer *et al.*, 1999; Boyer and Runyan, 2001; Brown *et al.*, 1996, 1999). There is a temporal separation for the requirements for TGFβ ligand signaling. TGFβ2 appears to mediate initial cell–cell separation of activated canal endocardium derived from chick embryos, while TGFβ3 is essential for subsequent mesenchymal cell formation and invasion into the underlying matrix. These data are consistent with a previously proposed model where TGFβ2 and TGFβ3 are sequentially and separately involved in the process of endocardial cushion transformation during avian embryogenesis (Boyer *et al.*, 1999). The role of TGFβs in mouse endocardial cushion formation has not been clearly defined. Nakajima *et al.* (1997) examined the roles of TGFβ molecules and latent TGFβ binding protein-1 in mouse AVC cushion explants. Although this investigation did show that concomitant neutralization of TGFβ1, TGFβ2, and TGFβ3 reduced mesenchyme formation by 73%, the study failed to elucidate functional specificity of particular TGFβ ligand(s) due to cross-reacting antibodies. Using specific and defined antibodies to TGFβ2, we show TGFβ2 functioning during mouse endocardial cushion EMT.

Our observations that mouse endocardial cells from AV canal explants do not normally display intermediate cell-to-cell separation prior to transformation, along with the differential expression patterns for *TGFβ2* and *TGFβ3* in the forming cardiac cushions, support an extended and distinct temporal requirement for TGFβ2, but not TGFβ3, for transformation and invasion events. Although *TGFβ2* and *TGFβ3* are both expressed in the avian cushion myocardium (Boyer *et al.*, 1999), we detect expression of only *TGFβ2* in the mouse AV canal cushion myocardium at a

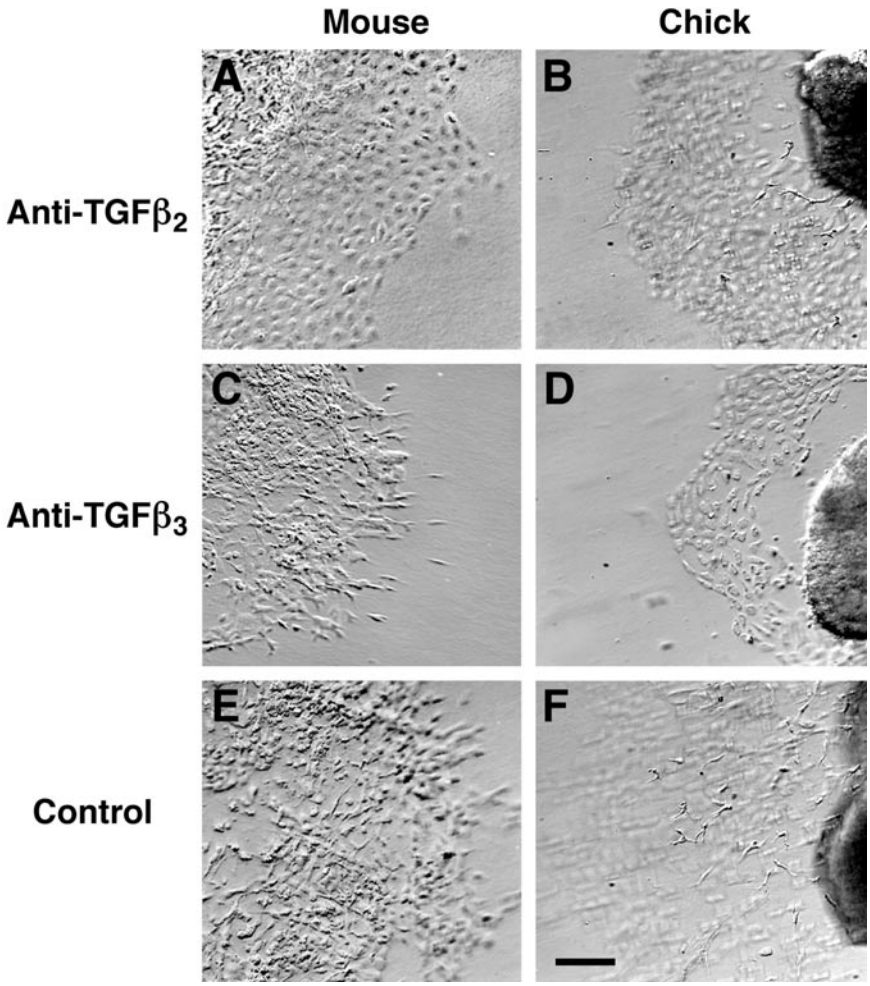

FIG. 4. TGFβ blocking antibody effects on E9.5 mouse and stage 15–16 chick AV canal explant cultures. TGFβ2 antisera inhibits mesenchyme formation in mouse AV canal explants (A); in chick explants, TGFβ2 antisera causes a lack of endothelial cell separation and decreased mesenchyme (B). Anti-TGFβ3 antibody-treated mouse AV canal explants (C) are indistinguishable from control mouse explant cultures (E); in chick AV canal explants, TGFβ3 antisera has a profound effect on cell transformation, but not cell–cell separation, (D). Optical section of a control chick explant demonstrates invasive mesenchyme and cell separation in the overlying endothelial sheet (F) similar to mouse control explants in (E). Scale bar, 40 μm.

time coincident with endocardial cell transformation (~E9.5) (Fig. 2; and see Fig. 6). The expression of *TGFβ2* persists through the duration of formation of the mitral and tricuspid valves (data not shown). The observation that *TGFβ2* is detected in both the cushion myocardium as well as in transformed mesenchymal cells suggests that it may serve an autocrine function in addition to its paracrine pathway (Dickson *et al.*, 1993). In contrast, *TGFβ3* does not become detectable until post-transformation and invasion events in the mouse (Figs. 2 and 3). The expression pattern for *TGFβ3* appears to be more relevant in later stages of AVC morphogenesis and in major vessel formation (Fig. 3C). This postulate was confirmed by demonstrating a dependence only on TGFβ2, independent of TGFβ3, for mesenchyme formation in mouse AV canal explants.

These expression and function data examining early EMT in mouse and chick AVC explants appear to correlate with mouse embryos deficient for TGFβ2. Recent detailed examination of TGFβ2 knockout mice beyond E14 showed hypoplasia of the septal contribution of the AV canal endocardial cushions (Bartram *et al.*, 2001). Such an abnormality results in incomplete endocardial cushion fusion and ventricular septal defect. Also observed were abnormal endocardial cushion volume related to delayed myocardialization of the cushions compromised by thickening of the primitive valve leaflets and retention of a cushion like

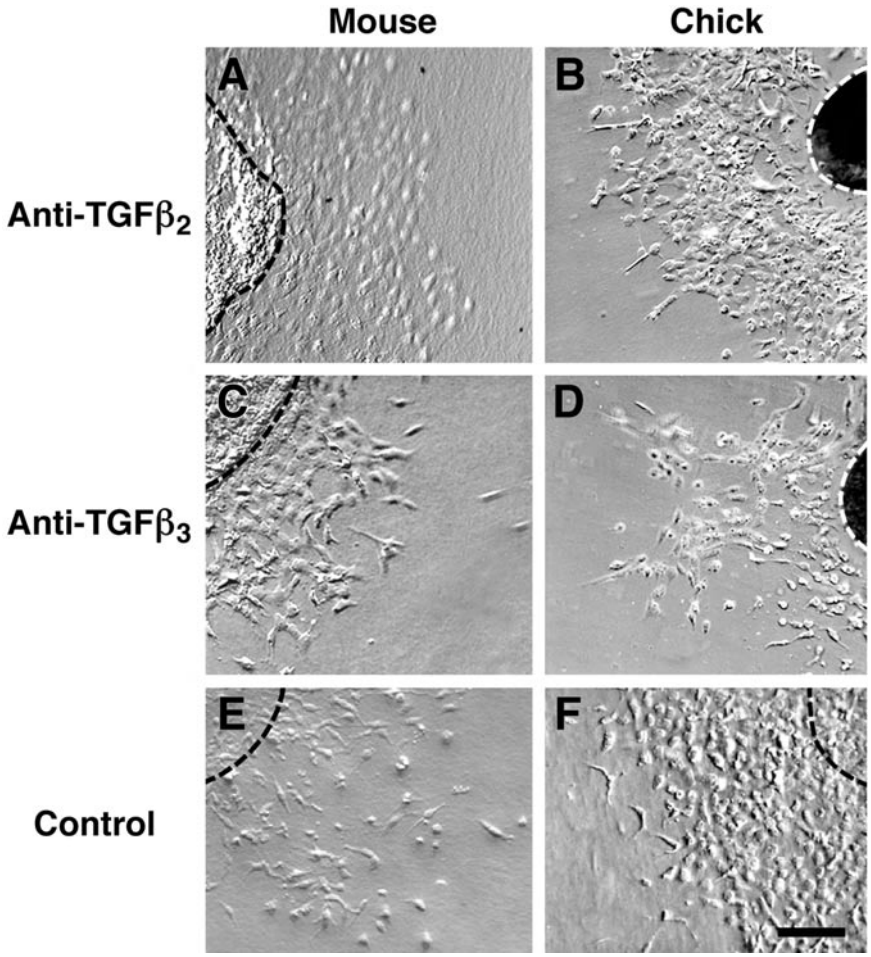

FIG. 5. TGFβ blocking antibody effects on outflow tract explant cultures. TGFβ2 antiserum inhibits cell transformation in mouse outflow tract cultures (A), but has little effect on cell–cell separation or transformation in avian outflow tract explants (B). In contrast, TGFβ3 antiserum has no significant effect on mouse outflow tract cushion explants (C) but causes an inhibition of cell transformation in chick explants (D). Normal outflow tract endocardial cushion explants are shown for mouse (E, ~E9.5) and chick (F, stage HH15–HH16). Scale bar, 40 um.

appearance in older TGF$\beta2^{-/-}$ embryos (~E14). These findings suggest endocardial cushion cell differentiation, rather than physical cushion formation, is perturbed in the absence of TGFβ2. However, in the mouse, we and others (Dickson *et al.*, 1993) show that TGFβ2 is expressed in the forming heart prior to AV valve formation and remodeling

TABLE 3
Summary of TGFβ Ligand Requirements during Mouse and Avian Endocardial Cushion Morphogenesis *in Vitro*

	Mouse		Chick	
Condition	Activation	Transformation	Activation	Transformation
No treatment	N/A*	+	+	+
+TGFβ_2 blocking Ab	+/−	↓	↓	−
+TGFβ_3 blocking Ab	N/A*	+	+	↓

Note. +, normal; ↓, inhibited; N/A, not applicable; *, not readily defined in E9.5 mouse endocardial cushion explant cultures applicable; +/−, intermediate endocardial cell phenotype.

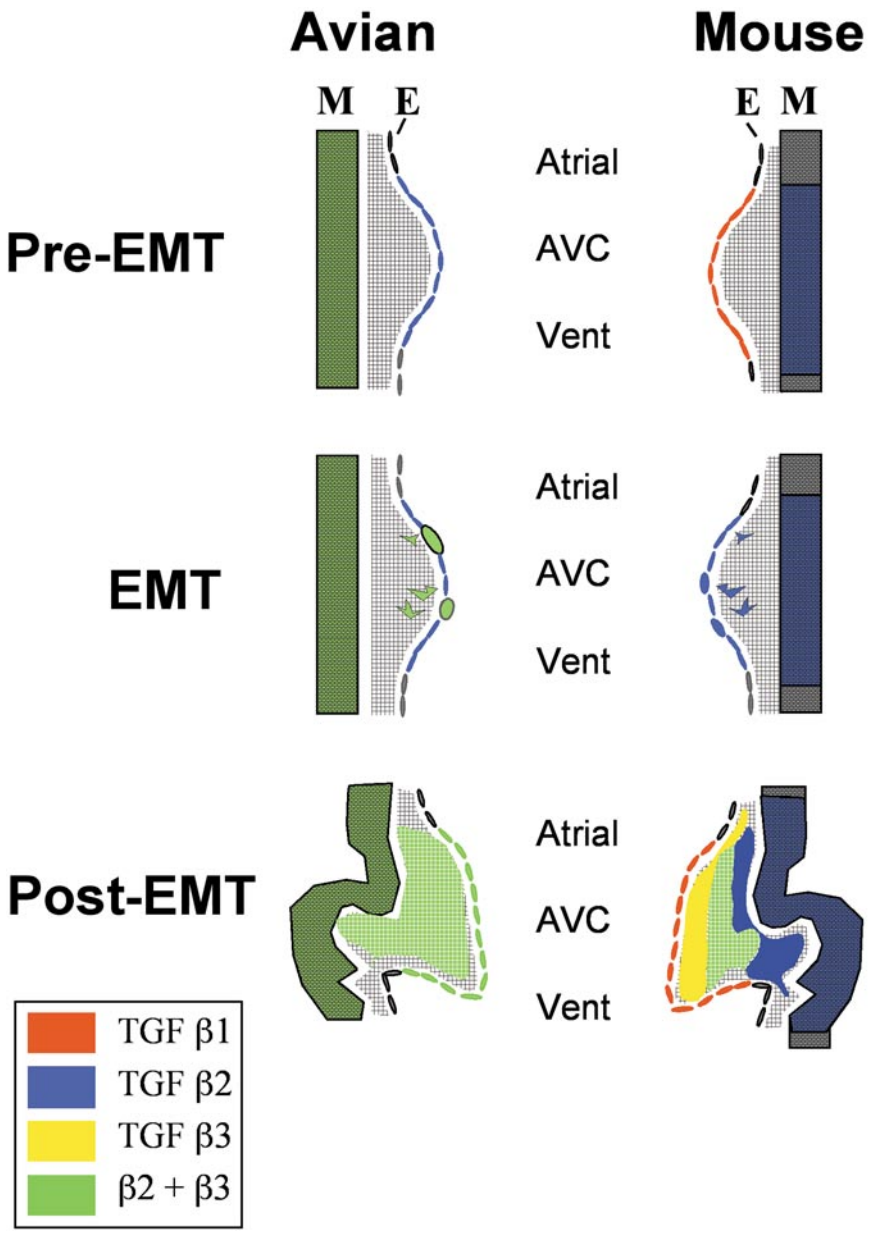

FIG. 6. Model of expression-function for TGF*β* ligands during AV canal morphogenesis. Pre-EMT, EMT, and post-EMT stages for AV canal development are sequentially shown from top to bottom. TGF*β* molecules at each stage and tissue are specified by color for both avian and mouse tissue from this report and those referenced within. Atrial, atrium side of canal; AVC, atrioventricular canal; Vent, ventricle side of canal; M, myocardium; E, endocardium.

(summarized in Fig. 6). These data support the hypothesis that TGF*β*2 is not a sole inducer of morphologic events during mouse AV canal development, but it is a key participant. Given that the activin receptor-like kinase 2 (ALK2) has been shown to mediate transformation in endocardial cushions (Lai *et al.*, 2000), it is possible that TGF*β*3 signaling may act cooperatively with other TGF*β* superfamily members, including BMP4 (Winnier *et al.*, 1995) or BMP2 (Yamagishi *et al.*, 1999), to promote transformation of cushion endocardium into mesenchyme. In this regard, BMP2 has very strong and specific expression to the AV canal myocardium at E9.5 (Lyons *et al.*, 1990); however, in mouse embryos lacking BMP2 (Zhang and Bradley, 1996), the heart tube forms in the exocoelomic cavity and not

inside the amniotic cavity, leaving only speculation as to a direct role for BMP2 during cardiac valve morphogenesis.

The observed differences in temporal expression profiles and *in vitro* functional activities for TGFβ2 and TGFβ3 are consistent with a dual requirement for both growth factors in the developing cardiac endocardial cushions for both mouse and avian species. Although we have demonstrated that TGFβ3 is not required for cushion mesenchyme formation in the mouse, its expression in the endocardial cushions at and beyond E12 suggests that it is involved in subsequent valve leaflet remodeling events. These data may account for the morphological variation between avian and mammalian valve structures as well as in the outcomes in the collagen gel assays. The prototypical member of this growth factor family, TGFβ1, is more important for regulation of chronic vascular diseases and not cardiovascular development (Topper, 2000). Although neither TGFβ1- nor TGFβ3-deficient embryos exhibit cardiac phenotypes (Dickson *et al.*, 1995; Sanford *et al.*, 1997; Taya *et al.*, 1999), TGF$\beta2^{-/-}$/TGF$\beta3^{-/-}$ compound homozygote embryos display a more severe cardiac defect phenotype and die earlier than TGF$\beta3^{-/-}$ embryos (M. Azhar and T. Doetschman, personal communication). Collectively, these observations indicate cooperative roles for TGFβ mediators during endocardial cushion formation, transformation, and post-transformation events, including valve differentiation and septation.

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